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Identification of the Active Precipitin Components in a Purified Preparation of the A Antigen of *Blastomyces dermatitidis*

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A purified A-antigen preparation of *Blastomyces dermatitidis* was determined to be composed of five major glycoprotein bands, visible with Coomassie blue and periodic acid-Schiff staining of polyacrylamide gels. At least 20 additional protein bands were detected by using a silver stain, which was 100 times more sensitive than the Coomassie method. Two components of this mixture were determined to be associated with the A-antigenic activity of *B. dermatitidis*. Of several antigen preparations examined in Ouchterlony precipitation tests, those reactive with a reference anti-A antiserum contained the slowest moving of the Coomassie blue bands. The antigen preparations without precipitin reactivity lacked this protein band. Two protein bands were shown to disappear from an antigen preparation after incubation with an affinity gel linked to the reference anti-A serum. One of the bands was the slowest Coomassie blue band, and the other was a fast-migrating protein detectable only with the silver stain. Characterization of the components responsible for the A-antigenic activity has important applications in the production and standardization of serological reagents for the diagnosis of blastomycosis.

The A antigen of *Blastomyces dermatitidis* was first described in 1973 by Kaufman et al. (11), who found the activity in yeast broth filtrates. The antigen was subsequently used to identify *B. dermatitidis* with 100% accuracy while giving no cross-reactions to various other fungi (12). A reference antiserum developed in 1979 (8) aided Green et al. in using anion-exchange chromatography to purify an antigen fraction showing only A banding in Ouchterlony tests (9). In this form, the antigen also showed promise as a serological tool in identifying active cases of blastomycosis by complement fixation, skin testing, enzyme-linked immunosorbent assay (ELISA) procedures, and Ouchterlony methods (9).

The continuing bane of fungal antigens as serological tools has been a combination of low sensitivity and high cross-reactivity. Although no cross-reactions with other fungi have been reported when the A antigen is used in Ouchterlony tests, Green et al. (8) reported that such testing was positive in only 52% of known human cases. The same problem arose with complement fixation, where only 70% of the cases gave positive results. And although the ELISA procedure detected 92% of known cases, it had a high (20%) rate of cross-reacting with various other fungal disease sera (9).

Crude extracts and impure antigen preparations have long been used in fungal serological and epidemiological studies. Such preparations have been shown to contain high numbers of common antigenic components which complicate the interpretation of immunological tests (1, 3, 10). It is possible that the usefulness of the A antigen of *B. dermatitidis* is still suboptimal because of contaminants in even the most recent pure preparations. It is, therefore, the purpose of this paper to more exactly identify those components associated with the A-antigenic activity. Gel electrophoresis, affinity chromatography, and comparisons of various antigenic preparations are presented to show that two specific proteins or glycoproteins can be identified as the active components of the A antigen. The presence of these proteins in polyacrylamide gels can thus be used as a marker to identify antigen preparations which contain the A antigen and as a standard against which the purity of various preparations may be measured.

MATERIALS AND METHODS

Antigen preparation. Cell cytoplasmic and cell wall supernatant antigens were prepared by inoculat-

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ing 1-liter amounts of brain heart infusion broth with a log-phase culture of the respective isolate and incubating for 3 days on a rotary shaker. The cells were killed by adding either formaldehyde (0.2%, final concentration) or merthiolate (0.05%, final concentration) and refrigerating for 48 h. Whole cells were collected by centrifugation at 5,000 × *g* for 10 min and were washed three times with distilled water. A 50% suspension of cells was made in distilled water, added to an equal volume of 0.45- to 0.50-mm-diameter glass beads, and broken for 90 s in a CO_2-cooled Braun homogenizer. Cell debris was removed by centrifugation at 20,000 × *g* for 30 min, and the supernatant was designated the cytoplasmic antigen. The cell wall debris was washed three times with distilled water, suspended 1:1 in distilled water, and stored at 4°C for at least 4 weeks. The water-soluble portion of this preparation was designated the cell-wall supernatant antigen. The cytoplasmic antigen from one isolate (SCB-2) was precipitated by adding either 1 or 2 volumes of 95% ethanol to give a 50 and 67% ethanol precipitate, respectively.

Table 1 lists the antigens used. The OU series was prepared in this laboratory. All other antigens were prepared and supplied by Sharon Harris and Stan Bauman of Immunomycologics, Inc., Norman, Okla. The Marshfield isolates are clinical isolates from Marshfield, Wis. The SCB and Davis isolates are also clinical isolates. All but two of the antigens, OU-S/CW and OU-D/CW, are cytoplasmic preparations. Purified A antigen and reference A antiserum were supplied by W. K. Harrell, Centers for Disease Control, Atlanta, Ga. The preparation and purification of this antigen are described by Green et al. (9).

Ouchterlony immunodiffusion. Immunodiffusion was performed in prepared slides from Immunomycologics, Inc. Rabbit anti-mouse immunoglobulin G (IgG), anti-mouse IgM, and pure mouse IgG and IgM from myeloma lines MOPC-21 and MOPC-104E were obtained from Bionetics Laboratory Products.

PAGE. Polyacrylamide gel electrophoresis (PAGE) was performed in an apparatus constructed according to the instructions of Ogita and Markert (15). The exact procedures and formulations of all solutions used in this study are tabulated in the same paper (15). Only two alterations were made. First, glycerol was omitted from the acrylamide-bisacrylamide solutions; second, 1 ml of 1% bromphenol blue was added directly to the upper reservoir instead of to each sample dilution. Running gels of 8, 14, or 20% acrylamide-bisacrylamide were overlaid with a 4% stacking gel. Gels were electrophoresed until the dye front was between 3 and 5 mm from the bottom edge of the gel.

Proteins were visualized in the gels by staining for 1 h in 0.1% Coomassie blue R-250 in 50% trichloroacetic acid. Gels were then destained in 10% acetic acid for 1 to 2 h at 60°C or for 24 to 48 h at 25°C. In selected cases, proteins were stained by the modified silver stain of Oakley et al. (14).

Gels were stained for carbohydrates by the periodic acid-Schiff method of Zacharius et al. (19).

Affinity chromatography. Antibodies were linked to Affi-Gel 10 (Bio-Rad) according to the manufacturer's directions. After prewashing, 30 to 40 mg of antibody was added to 4 to 5 ml of gel and allowed to react overnight at 4°C with mild agitation. The antibody-linked gel was poured into a 1-cm-diameter column; unabsorbed antigen was washed out with tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (0.1 M, pH 8.0, and 0.15 to 0.5 M NaCl) and allowed to stand in the same buffer for 1 h to saturate any remaining sites. The gel was washed with glycine-hydrochloride elution buffer (0.05 M, pH 2.4) and rewashed with Tris buffer. Antigen (1.3 to 1.5 mg in 2.5 ml of Tris) was added and incubated for 1 h at room temperature. Unadsorbed antigen was washed out with Tris buffer, and adsorbed antigen was removed by the glycine-hydrochloride buffer.

RESULTS

Ouchterlony precipitation with anti-A reference serum. Precipitin lines of identity were formed between the purified A antigen and the following antigen preparations: OU-S, OU-S/CW (slight), OU-D, OU-D/CW (very slight), 205B3 (slight), OU-S/50, and OU-S/67. Lines were absent in the following antigen preparations: 008BY, 009BY, 00AB3, 009B3, 007B3, and 008B3 (Fig. 1). Close inspection of the line formed midway between the purified A antigen and the anti-A reference serum revealed it to be composed of two very close lines which were fused. This could be most clearly seen in the photograph of the reactions involving the two ethanol precipitate antigens, although the same phenomenon seemed to occur with all reactive preparations (Fig. 1).

PAGE of purified A antigen. Purified A antigen was electrophoresed in 14 and 20% polyacrylamide gels and stained for protein by the Coomassie blue and silver methods (Fig. 2 and 3). Only five protein bands appeared when

TABLE 1. *Antigens*

Antigen	Isolate	Prepn[a]	Protein[b] (mg/ml)
OU-S	SCB-2	Formalin	1.0
OU-D	Davis	Formalin	1.4
008BY	CDC-A373	Merthiolate	2.0
009BY	CDC-A373	Merthiolate	1.0
205B3	Marshfield 1	Formalin	0.7
008B3	Marshfield 1	Formalin	0.6
007B3	Marshfield 1	Merthiolate	0.4
009B3	Marshfield 2	Formalin	1.1
00AB3	Marshfield 2	Merthiolate	0.5
OU-S/CW	SCB-2	Formalin, cell wall	ND[c]
OU-D/CW	Davis	Formalin, cell wall	ND
OU-S/50[d]	SCB-2	Formalin, EtOH-ppt[e]	ND
OU-S/67[d]	SCB-2	Formalin, EtOH-ppt	ND

[a] Method of killing. Unless otherwise stated, all are cytoplasmic preparations.

[b] Protein was measured by the method of Lowry as modified by Garvey et al. (7) or by the Coomassie blue dye-binding method of Bradford (2) as modified by Spector (18).

[c] ND, Not done.

[d] Designates the antigen fractions precipitated from the OU-S antigen by either 50 or 67% ethanol treatment.

[e] EtOH-ppt, Ethanol precipitate.

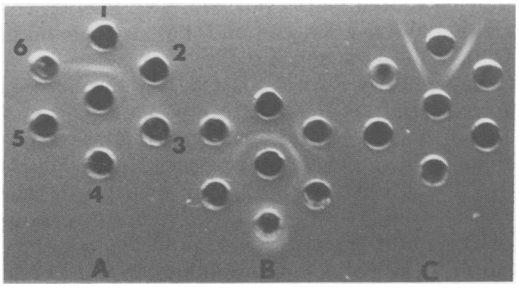

FIG. 1. *Precipitin bands between anti-A reference serum and various antigen preparations. The center wells of the A and B templates and well 1 of the C template contained anti-A serum. Template A tested these antigens: well 1, purified A antigen; 2, 205B3; 3, 00AB3; 4 009B3; 5, 007B3; 6, 008B3. Template B tested these antigens: well 1, purified A antigen; 2, OU-S; 3, OU-S/CW; 4, 009BY; 5, OU-D/CW; 6, OU-D. Template C tested: well 2, OU-S/50; 5, OU-S/67.*

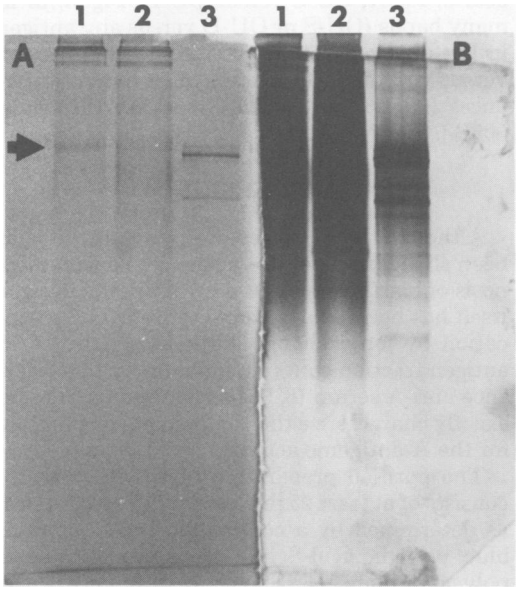

FIG. 2. *Protein stain of 14% gels. Track 1, OU-S; 2, OU-S after passage through affinity column containing anti-A antigen reference serum; 3, purified A antigen. Gel A, Coomassie blue stain; gel B, silver stain. Arrow locates common protein band which is partially removed by affinity chromatography from the OU-S preparation in track 2, gel A.*

stained with Coomassie blue, with R_f values between 0.19 and 0.29 in the 14% gel and between 0.10 and 0.17 in the 20% gel. The silver stain revealed a minimum of 25 well-defined protein bands, with the most rapidly moving band having an R_f of 0.54 and 0.27 in the 14 and 20% gels, respectively. Periodic acid-Schiff staining revealed five carbohydrate bands corresponding to the five seen with Coomassie blue. The silver stain also revealed numerous additional bands in the OU-S preparation, which reproduced photographically as an almost solid black track.

Affinity chromatography. The removal of protein bands from OU-S by affinity chromatography through an anti-A-linked gel is shown in Fig. 2 and 3. In both figures, the nonadsorbed OU-S had been adjusted to the same protein concentration as the applied OU-S. Figure 2 most clearly shows the reduction of the slowest-moving band of the A antigen from OU-S (Coomassie blue-stained gel). This band is one shown to be common to both preparations (Fig. 4 and 5). An additional, rapidly migrating protein band, which appeared only after silver staining, was completely absent from the antigen exposed to the affinity gel but was present both in the OU-S antigen before application to the gel and in the purified A antigen (Fig. 3).

Comparison of purified A antigen with other preparations. Gel scans of polyacrylamide gels are shown in Fig. 4 and 5. These gels compare the purified A antigen with the OU-S preparation, which reacted with anti-A serum in Ouchterlony tests, and with the 008BY preparation, which was nonreactive. Both the 14 and 20% gels revealed only one common band between the reactive OU-S preparation and the purified A antigen. This particular band ap-

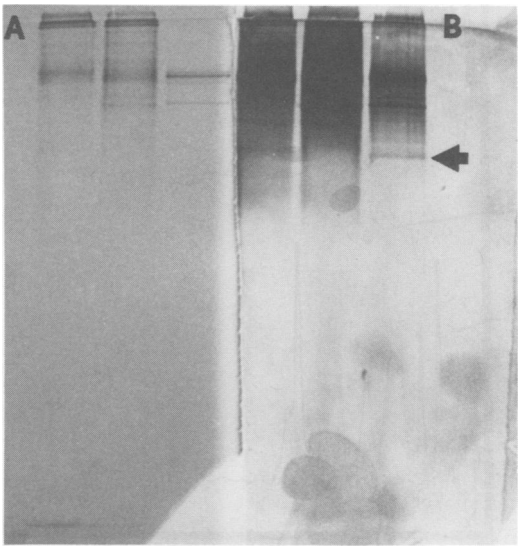

FIG. 3. *Protein stain of 20% gels, arrangement as in Fig. 2. Arrow locates common protein band removed by affinity chromatography from the OU-S preparation in track 2, gel B.*

peared only faintly, if at all, in the nonreactive 008BY preparation, and then only in the 20% gel. The correspondence between the slowest-moving protein of the purified A antigen (in the Coomassie-stained gels) and the largest band in OU-S can be seen in Fig. 2.

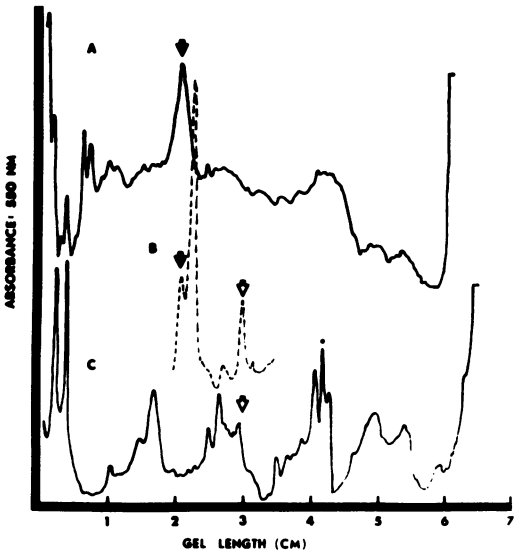

FIG. 4. *Gel scan of 14% gels stained with Coomassie blue. Antigen preparations are: A, OU-S; B, purified A antigen; C, 008BY. Arrows designate bands held in common.*

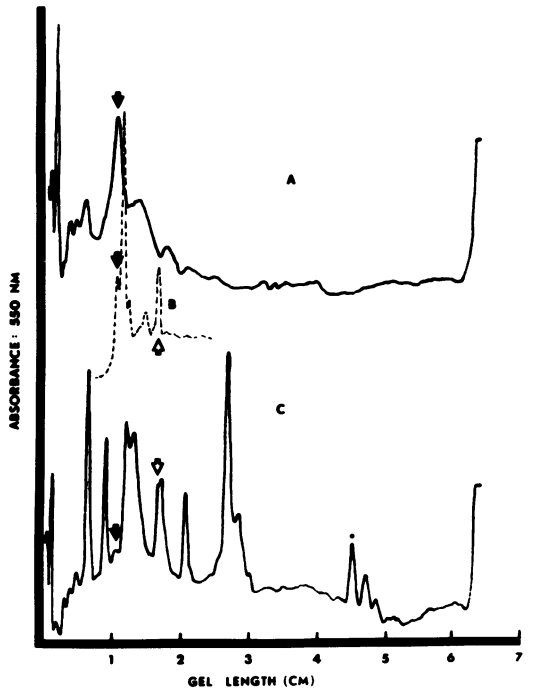

FIG. 5. *Gel scan of 20% gels stained with Coomassie blue. Details as in Fig. 4.*

Several other antigens were electrophoresed to compare the presence or absence of this particular band with the reactivity of the preparations in Ouchterlony precipitation tests (Fig. 6–8). In all cases, presence or absence of the marked band paralleled the presence or absence of precipitin reactivity to the anti-A reference serum. The corresponding bands in OU-D and 205B3 in Fig. 6 were much lighter than in OU-S and were not clearly reproduced photographically. The A-antigenic activity was shown to be precipitated with ethanol treatment (Fig. 8).

In addition, these results demonstrate that lot-to-lot variation of antigen preparations may be absent (008BY versus 009BY, Fig. 7) or slight (007B3 versus 00AB3, Fig. 7). Strain-to-strain variations may occur in relatively few protein bands (007B3 versus 00AB3 and 008B3 versus 009B3, Fig. 7; OU-S versus OU-D, Fig. 6) or in many bands (OU-S or OU-D versus any antigen in Fig. 7). Preparation by killing with Formalin versus merthiolate yielded only minor differences (007B3 versus 008B3 and 00AB3 versus 009B3, Fig. 7).

DISCUSSION

Although the A antigen of *B. dermatitidis* has been shown to be of use in the serological diagnosis of blastomycosis (9, 11, 12, 16), the antigen itself has been poorly characterized. The purification by ion-exchange chromatography of an antigen fraction containing reactivity to a reference anti-A serum (8, 9) has allowed us to more exactly characterize the components responsible for the A-antigenic activity.

The purified preparation of Green et al. (9) consists of at least 25 proteins and glycoproteins, as determined by a combination of Coomassie blue, periodic acid-Schiff, and silver staining of polyacrylamide gels. Such a mixture may be the reason that cross-reactions with other mycotic disease sera continue to occur in the highly sensitive ELISA procedure (9). The use of a mixture may be appropriate in less sensitive techniques, such as Ouchterlony precipitation, but an unacceptable increase in false-negative reactions also occurs (9). As long as extraneous material is included in antigen preparations, the absence of cross-reactions cannot be guaranteed.

Although previous reports suggest that precipitation tests yield a single band between the A antigen and anti-A serum, we have noted that the reaction is more likely one giving two fused bands. This correlates well with the finding that

two specific components are involved in the A-antigenic activity.

In the past, Ouchterlony tests have been the primary method used to assay various antigen fractions for the presence of the A antigen. Although this may be useful as a preliminary test, a more sensitive assay is needed if very pure preparations are to be produced. Huppert et al. (10) have suggested the use of two-dimensional electrophoresis as a tool for both standardizing and assessing the purity of antigen preparations. The major drawbacks to this use of the method include the amount of time and material required to perform the test, its relative insensitivity, and its reliance on an antigen-antibody reaction. This latter is restricting, since a large quantity of antibody must be used and antigens of possible significance may go undetected if specific antibody is unavailable. The staining of proteins in polyacrylamide gels is a rapid and very sensitive procedure, especially if the new silver stain is used, and many samples may be compared in detail. For comparative purposes, a standard is now available using the purified A antigen with knowledge of its specific reactive components.

The location of the A antigen has also been addressed. Kaufman et al. (11) first located it in yeast culture filtrates. It has since been most commonly prepared by extracting whole yeast cells with water or saline over a several-week period (8, 9, 12). Reference anti-A serum has been reported to be adsorbed by yeast cell walls but not by cytoplasmic preparations (9). These previous investigations have been performed with only two isolates of *B. dermatitidis*, A295 and A373. We have verified that the A antigen is absent, or present in insignificant amounts, in the cytoplasm of A373 (antigens 008BY and 009BY). However, other isolates have shown significant amounts of A antigen in cytoplasmic preparations (OU-S and OU-D). This has two ramifications. First, preparation of large quantities of the A antigen can be made more quickly by breaking whole cells, although contamination with extraneous material may be greater than in aqueous extracts. Second, the precise location and function of the A antigen remains in doubt.

The finding that the A antigen can be precipitated by ethanol may have some historical, and perhaps practical, implications. As early as 1911, Davis (4) isolated a fraction from yeast cytoplasm by ethanol precipitation which yielded a fairly sensitive precipitin antigen for infected guinea pig serum. Many investigators have since used ethanol precipitation to isolate surprisingly sensitive antigens (5, 6, 13, 17). None of these preparations was characterized in depth, and the possibility that they contained what is now

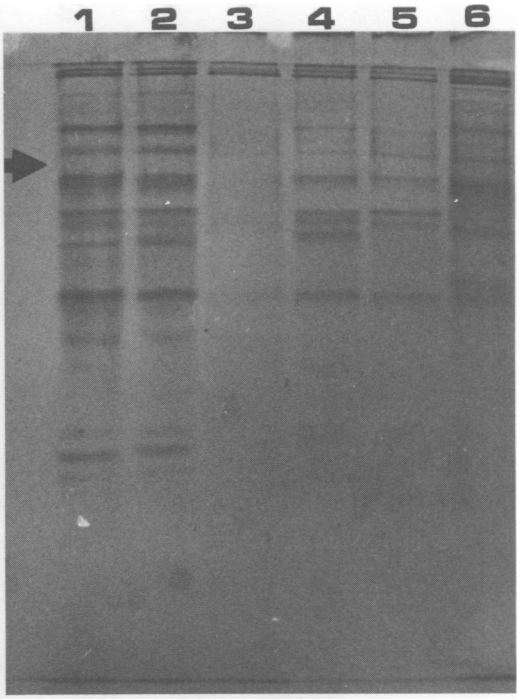

FIG. 7. *Coomassie blue stain of 20% gel. Track 1, 008BY; 2, 009BY; 3, 00AB3; 4, 009B3; 5, 007B3; 6, 008B3. Arrow locates the position at which the OU-S/A-antigen common band should appear.*

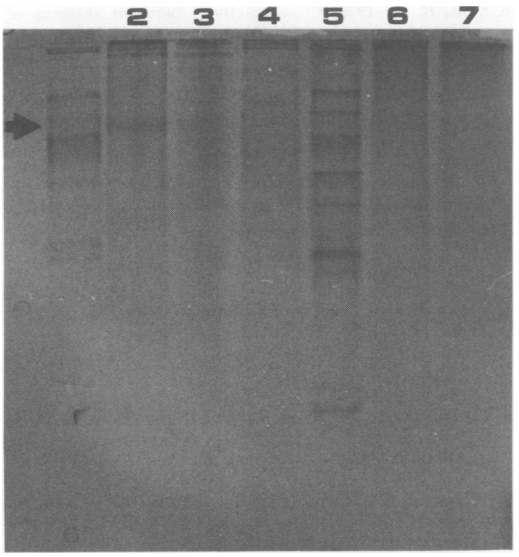

FIG. 6. *Coomassie blue stain of 20% gel. Track 2, OU-S; 3, OU-D; 4, 205B3; 5, 008BY; 6, OU-S/CW; 7, OU-D/CW. Arrow locates position of the OU-S/A-antigen common protein band.*

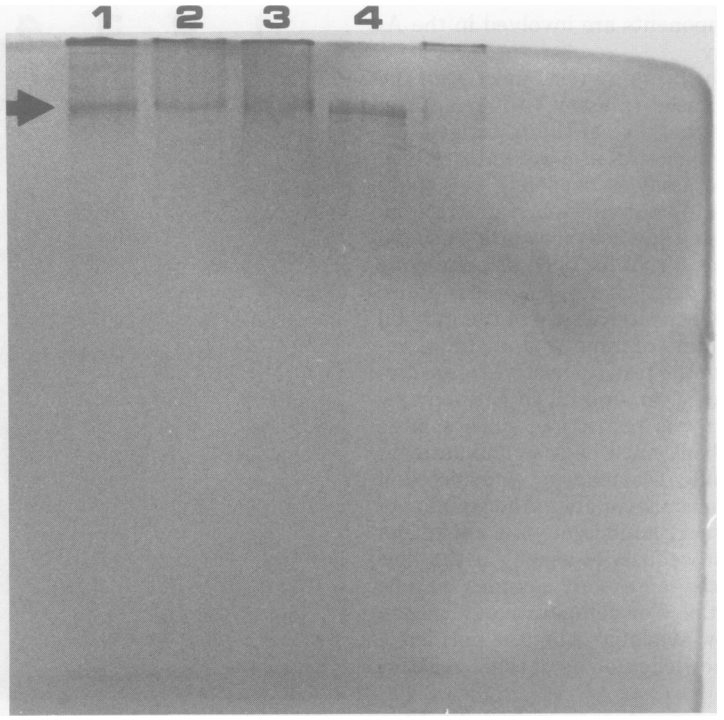

FIG. 8. *Coomassie blue stain of 20% gel. Track 1, OU-S; 2, OU-S/50; 3, OU-S/67; 4, purified A antigen. Arrow locates the OU-S/A-antigen common protein band.*

termed the A antigen seems quite high. Incorporation of an ethanol precipitation step may therefore be useful in future purification schemes.

Rippon et al. have noted that without the tools of skin test or complement fixation antigens for epidemiological, diagnostic, and prognostic use, "we are at least 50 years behind in our defining the disease blastomycosis" (17). Much of the problem has been due to impure antigen preparations and an imprecise understanding of the reactive agents. Hopefully, the identification of particular reacting components will help alleviate some of the problems involved in closing such a large gap.

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